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[TRANSLATION]

DESCRIPTION

METHOD FOR MICROBIAL PRODUCTION OF AMINO ACIDS OF THE ASPARTATE
 AND/OR GLUTAMATE FAMILY AND AGENTS WHICH CAN BE USED IN SAID METHOD

Field of the Invention

The invention relates to a method of microbial production
 of amino acids of the aspartate family and/or of the glutamate
 family ~~according to claims 1 to 17~~, to the pyruvate-carboxylase
 gene ^{and to} ~~according to claims 18 to 23~~, gene structures ~~according to~~
 claim 24, vectors ^{and} ~~according to claim 25~~, transformed cells
^{containing the pyruvate carboxylase gene.}
~~according to claims 26 to 31 as well as to uses according to claims~~
~~32 to 37~~

Background of the Invention

Amino acids are of considerable economic interest since
 amino acids have many uses: thus, for example, L-lysine and L-
 threonine, L-methionine and L-tryptophan are necessary as fodder
 additives, L-glutamate as an additive to suppress L-isoleucine and
 L-tyrosine in the pharmaceutical industry, L-arginine and L-
 isoleucine as medicaments or L-glutamate, L-aspartate and L-
 phenylalanine as starting substances for the synthesis of fine
 chemicals.

A preferred method of producing these different amino
 acids is the biotechnical production by means of microorganisms
 such that in this manner the biologically-effective and optically-
 active forms of the respective amino acids are obtained and simple

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and inexpensive raw materials can be used. As microorganisms, for example, *Corynebacterium glutamicum* and its derivatives *ssp. Flavum* and *ssp. Lactofermentum* (Liebl et al., Int J System Bacteriol 1991, 41: 255 to 260) in addition to *Escherichia coli* and related
5 bacteria are used. These bacteria normally produce the amino acids but only in amounts required for growth so that no surplus amino acids are formed and can be recovered. This is because in the cells the biosynthesis of amino acids is controlled in many ways. As a consequence, there are already known various processes to
10 increase the product formation by cutting out the control mechanisms. In these processes, for example, amino acid analogs are introduced to switch off the effective regulation of the biosynthesis. For example, a process has been used which is resistant to L-tyrosine analogs and L-phenylalanine analogs (JP
15 19037/1976 and 39517/1978). The processes also have been described in which bacteria resistant to L-lysine analogs or L-phenylalanine analogs have been used to suppress the control mechanisms (EP 0 205 849, GB 2 152 509).

Furthermore, microorganisms which have been constructed
20 also by recombinant DNA-techniques ~~which also~~ obviate regulation of biosynthesis in that the gene which is coded in the no-longer feedback-inhibited key enzyme is cloned and expressed. For example, the recombinant L-lysine-producing bacterium with plasmid-coded feedback-resistant aspartate kinase is known (EP 0 381 527).
25 In addition, a recombinant L-phenylalanine-producing bacterium with

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feedback-resistant prephenate dehydrogenase is described (JP 123475/1986, EP 0 488 424).

In addition, by overexpression of genes which do not code for feedback-sensitive enzymes ⁱⁿ ~~as~~ amino acid synthesis, increased amino acid yields are obtainable. Thus, for example, ~~a~~ lysine formation can be improved by increased synthesis of the dihydrodipicolinate synthesis (EP 0 197 335). Increasingly, by increased synthesis of the threoninedehydratase, improved isoleucine formation is achieved (EP 0 436 886).

Further investigations in increasing amino acid production have been targeted on the improved availability of the cellular primary metabolites of central metabolism. Thus it is known that, by recombinant techniques, over-expression of the transketolase can bring about an improved product formation of L-tryptophan or L-tyrosine or L-phenylalanine (EP 0 600 463). Furthermore, ^a ~~the~~ reduction of the phosphoenolpyruvate-carboxylase activity in Corynebacterium leads to improved formation of aromatic amino acids (EP 0 3331 145) whereas by contrast ^{an} ~~the~~ increase in the phosphoenolpyruvate-carboxylase activity in Corynebacterium leads to increased ^{recovery} ~~(separation)~~ out of amino acids of the aspartate family (EP 0 358 940).

During the growth and especially under amino acid production conditions, the tricarboxylic acid cycle must continuously and effectively be supplemented with C4 compounds, for example, oxalic acetate to replace intermediate products withdrawn for the amino acid biosynthesis. Until recently it has been

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thought that phosphoenolpyruvate-carboxylase was ^{responsible} answerable for these so-called anaplerotic functions in *Corynebacterium* (Kinoshita, *Biology of Industrial Micro-organisms* 1985: 115 to 142, Benjamin/Cummings Publishing Company, London; Liebl, *The Prokaryotes II*, 1991 to 1171, Springer Verlag N.Y.; Vallino and Stephanopoulos, *Biotechnol Bioeng* 1993, 41: 633 to 646).

It has, however, now been found that phosphoenolpyruvate-carboxylase-negative mutants grow equally by comparison to the respective starting strains on all media (Peters-Wendisch et al., *FEMS Microbiology Letters* 1993, 112: 269 to 274; Gubler et al., *Appl Microbiol Biotechnol* 1994, 40: 857 to 863). These results indicate that the phosphoenolpyruvate-carboxylase is not essential for the growth and plays no role or only a small role for the anaplerotic reactions. Furthermore the aforementioned results indicate that in *Corynebacterium* another enzyme must be provided which is ^{responsible} answerable for the synthesis of oxalacetate which is required for ~~the~~ growth. Recently, indeed, a pyruvate-carboxylase activity has been found in permeablized cells of *Corynebacterium glutamicum* (Peters-Wendisch et al., *Microbiology* 1997, 143: 1095 to 1103). This enzyme is effectively inhibited by AMP, ADP and acetyl coenzyme A and in the presence of lactate as a carbon source is formed in increased quantities. Since one must conclude that this enzyme is answerable primarily for the satisfaction of the tricarboxylic acid cycle of growth, it was to be expected that an increase in the gene expression or the enzymatic activity would either give rise to no increase in the amino acids belonging to the

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aspartate or yield only an increase therein. Furthermore, it was to be expected that an increase in the gene expression or the enzymatic activity of the pyruvate-carboxylase would also have no influence on the production of amino acids of other families.

Summary of the Invention

5 It has surprisingly been found that an increase in the pyruvate-carboxylase activity by genetic modification of the enzyme and/or by increasing the pyruvate-carboxylase gene expression, the microbial production of amino acids of the aspartate and/or the glutamate families can be increased. It has been found that
10 especially strains with increased copy numbers of the pyruvate-carboxylase gene can produce about 50% more lysine, 40% more threonine and 150% more homoserine in the culture medium. It has been found further that, surprisingly, the glutamate production is also significantly increased (compare especially the example under
15 6. Table 4).

The genetic alteration of the pyruvate-carboxylase to increase the enzyme activity is effected preferably by mutation of the endogenous gene. Such mutation can either be achieved by classical methods like, for example, by UV irradiation or by
20 mutation triggering the chemicals or targeted by means of gene technological methods like deletion, insertion and/or nucleotide exchange.

The pyruvate-carboxylase gene expression is increased by increasing the gene copy number and/or by reinforcing regulatory
25 factors which positively influence the expression of the gene. Thus a reinforcement of regulatory elements, preferably on the

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transcription plane can be effected in that especially the transcription signals are increased. This can be effected, for example, by varying the promoter sequence of the promoter preceding the structure gene to enhance its effectiveness or by replacing the promoter completely by more effective promoters. A reinforcement of the transcription can also be effected by a corresponding influence on a regulator gene associated with the pyruvate-carboxylase gene. This can be achieved, for example, by mutation of a regulatory gene sequence to influence the effectivity of the binding of a regulator protein to the DNA of the pyruvate-carboxylase gene which is regulated so that the transcription is thereby enhanced and thus the gene expression is increased. Furthermore the pyruvate-carboxylase gene can also be associated with a so-called "enhancer" as a regulatory sequence and which by means of an improved interchange between RNA polymerase and DNA also effects an increased pyruvate-carboxylase gene expression. However, a reinforcement of translations is also possible in that, for example, the stability of the m-RNA is improved.

For increasing the gene copy number the pyruvate-carboxylase gene is built into a gene construct or vector. The gene construct contains especially the regulatory sequences associated with the pyruvate-carboxylase gene, preferably those which reinforce the gene expression. For the incorporation of the pyruvate-carboxylase gene in a gene construct, the gene is progressively isolated from a microorganism strain of the *Corynebacterium* variety and is transformed in an amino-acid

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producing microorganism strain, especially *Corynebacterium* or in *Escherichia coli* or *Serratia marcescens*. For the process of the invention, especially genes from *C. glutamicum* or *C. glutamicum* ssp. *flavum* or *C. glutamicum* ssp. *lactofermentum* are suitable.

5 After isolation of the gene and in the in vitro recombination with known vectors (see for example Simon et al., Bio/Technology 1983, 1: 784 to 791; Eikmanns et al., Gene 1991, 102: 93 to 98), the transformation is effected in the amino-acid producing strain by electroporation (Liebl et al., FEMS Microbiology Letters 1991, 65: 10 299 to 304) or conjugation (Schäfer et al., J. Bacteriol 1990, 172: 1663 to 1666).

As the host strain preferably such amino-acid producers are used which have been deregulated in the synthesis of the corresponding amino acid and/or show an increased export carrier activity for the corresponding amino acid. Furthermore, such 15 strains are preferred which contain an increased number of such central metabolism metabolites as anticipated in the synthesis of the corresponding amino acid and/or strains which contain a reduced proportion of the central metabolism metabolites which do not participate in the synthesis of the corresponding amino acid, 20 especially metabolites which tolerate competitive reactions; i.e. such strains are preferred ^{with} which ~~by~~ synthesis paths competitive with the corresponding amino acid biosynthesis path runs with reduced activity. Thus, especially a *Coryne*-former 25 microorganism strain with reduced citrate synthase activity is

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suitable as a strain resistant to L-asparaginic-acid- β -methylester (AME) ~~is suitable~~ (EP 0 551 614).

After isolation, the pyruvate-carboxylase gene is obtained with nucleotide sequences which code for the amino acid sequence given under SEQ ID No. 2 or their allele variations or the nucleotide sequence of nucleotides 165 to 3587 according to SEQ ID No. 1 or a substantially identically-effective DNA sequence. The gene further contains a protein promoter of the nucleotide sequence of nucleotides 20 to 109 according to SEQ ID No. 1, a substantially identically effective DNA sequence. Allele variations or identically effective DNA sequences encompass especially functional derivations which are corresponding nucleotide sequences formed by deletions, insertions and/or substitutions of nucleotides whereby the enzyme activity or function remains or can even be increased. This pyruvate-carboxylase gene is preferably used in the process of the invention.

The pyruvate-carboxylase gene with or without the preceding promoter or with or without the associated regulator gene can be preceded by and/or followed by one or more DNA sequences so that the gene is contained in a gene structure.

The pyruvate-carboxylase gene is preferably preceded by the tac-promoter (lacI^0 -Gen) ~~with~~ which is associated especially with regulatory sequences.

By cloning the pyruvate-carboxylase gene, plasmids are obtained which contain the gene and are suitable for transformation to an amino acid producer. The cells obtained by transformation

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which preferably correspond to transformed cells of
Corynebacterium, contain the gene in replicatable form, i.e. in
additional copies on the chromosome, whereby the gene copies are
integrated by recombination at optional sites in the genome and/or
on a plasmid or vector.

Brief Description of the Drawings
Figure 1 is a physical map and restriction analysis of the cloned pyruvate
carboxylase encoding gene from C. Glutamicum ATCC 13032 in pUC18 resulting in the vector pUCpyc.
Example

1. Cloning the Pyruvate-Carboxylase Gene of Corynebacterium Glutamicum

Starting from conserved regions of all prior known
pyruvate-carboxylase-(pyc-) genes of *Saccharomyces cerevisiae* (J
Biol Chem 1988, 263: 11493-11497; Mol Gen Genet 1991, 229: 307-
315), Mensch (Biochem Biophys Acta 1994, 1227: 46-52), Maus (Proc
Natl Acad Sci, USA 1993, 90: 1766-1770), *Aedes aegypti* (EMBL-
GeneBank: Accession Nr. L36530) and from *Mycobacterium tuberculosis*
(EMBL-GeneBank: Accession Nr. U00024), PCR primer is synthesized
(MWG Biotech). The primer corresponds to the bases 810 to 831 and
1015 to 1037 of the pyc gene from M. tuberculosis. With this
primer, by means of PCR according to the standard method of Innis
et al (PCR protocols. A Guide to Methods and Applications, 1990,
Academic Press) for nongenerated homologous primer, a fragment
of about 200 bp of chromosomal DNA of C. glutamicum ATCC 13032 as
has been described by Eikmanns et al. (Microbiology 1994, 140:
1817-1828) is isolated following amplification. The size of 200 bp
corresponds to the expectation for the pyc gene. The PCR product
as described by Sanger et al (Proc Natl Acad Sci USA 1977, 74:

Figure 2 is a physical map of the expression vector pVWEX1 con-
taining the pyruvate carboxylase encoding gene from C. glutamicum 13032. Abbreviations:
pvc = pyruvate carboxylase, Atac-IPTG = inducible synthetic promoter at
trp (-35 box) and lac-promotor (-70 box) regions; lacIq = gene encoding the
repressor of lac operon, Kan = gene encoding the resistance to Kanamycin.

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5463-5467) was sequenced. The sequencing was carried out with fluorescence-marked ddNTPs with an automatic DNA sequencing apparatus (Applied Biosystems).

Starting from this DNA fragment of *C. glutamicum*, the following homologous oligonucleotides are produced:

pyc 1 5'- CGTCTTCATCGAAATGAAC-3' SEQ ID NO:3

pyc 2 5'- ACGGTGGTGATCCGGCACT-3' SEQ ID NO:4

The oligonucleotide is used as a PCR primer for isolating the probe for the gene of pyruvate-carboxylase (pyc) from *C. glutamicum*. The primer is introduced into a PCR reaction with chromosomal DNA from *C. glutamicum* and digoxigenine-marked nucleotides. The reaction is carried out in accordance with the instructions of the "PCR DIG Labeling Kits" of the firm Boehringer Mannheim. With this approach, a digoxigenine-marked DNA fragment is amplified which corresponds to the expected size of about 200 bp. The thus produced pyc probe is then used to identify, utilizing Southern-blot-hybridization, A DNA fragment in the chromosomal DNA of *C. glutamicum* on which the pyc gene is localized. For this purpose each 2 to 5 µg of chromosomal DNA from *C. glutamicum* WT is cleaved with the restriction enzyme HindIII, SphI, SalI, DdraI, EcoRI and BamHI and the obtained DNA fragments are correspondingly separated by size over 16 hours at 20 volts gel-electrophoretically in an 0.8% agarose gel. The DNA fragments found in the agarose gel are denatured by the Southern blot (J Mol Biol 1975, 98: 503-517) and subjected to the vacuum-supported separation with the VacuGene Blot Apparatus of Pharmacia LKB

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(Uppsala, Sweden) from the gene matrix transferred onto a nylon membrane (Nytran N13 of Schleicher and Schüll, Dassel, Switzerland), immobilized and the digoxigenine marker detected by means of NBT/X phosphate conversion with alkali phosphatases in this manner. ^{The p} Following chromosomal fragments hybridized with the pyc-DNA-probe can be detected: a 17 kb HindIII-fragment, a 6.5 kb SalI fragment and a 1.35 kb EcoRI fragment.

The 17 kb HindIII fragment was isolated and subcloned. For this purpose a cosmid gene bank of chromosomal DNA from *C. glutamicum* in cosmid pH C79 was used which represented the genome of *C. glutamicum* to 99% (Mol Microbiol 1992, 6: 317-326). The *E. coli* strain DH5 α was transformed with this gene bank by means of the CaCl₂ method of Sambrook et al (Molecular Cloning, A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press) and plated out to about 300 colonies per LB-agar plate with 50 μ g/l kanamycin (a total of 5000 colonies). Then the obtained transformed product was transferred on a nytran N13 filter and incubated for 5 minutes for alkali lysis of the cells and denaturing of the DNA on Whatmann paper soaked with 0.5 M NaOH and 1.5 M NaCl. The subsequent neutralization is effected with 1 M Tris/HCl pH 7.5 and 1.5 M NaCl. The subsequent neutralization is effected with 1 M Tris/HCl pH 7.5 and 1.5 M NaCl.

After incubation of the filter in 2 x SSC, the liberated DNA is fixed by UV radiation at 366 nm on the filter. Then the remaining cell fragments are removed by shaking in 3 x SSC, 0.1% SDS at 50°C. The filter in this form is used for the hybridiza-

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tion with a specific pyc probe as described by Southern (J Mol Biol 1975, 98: 503-517).

The 3 transformands were identified from the pyc probe hybridization. From these transformands the cosmid DNA was isolated by means of plasmid proportion in accordance with the alkali lysis method of Birnboim (Meth Enzymol 1983, 100: 243-255) and then tested by restriction and Southern blot analysis for the presence of the HINDIII fragments. The cosmid pH C79-10 which contains a 40 kb HINDIII transmission completely and was further analyzed. It showed that also after the restriction with the endonucleosis Sall and EcoRI the same hybridized fragments as in the chromosomal DNA, i.e. a 6.5 kb Sall- fragment and a 1.35 kb EcRI-fragment. The 17 kb HindIII-fragment was isolated by restriction from the cosmid and is ligated in the E. coli vector pUC 18, which is also cleaved with HindIII. A restriction analysis of the fragments in the resulting vector pUC pyc was carried out. The physical mapping of the fragments is shown in FIG. 1.

2. Sequencing of the Pyruvate-Carboxylase Gene

In further subcloning steps a 0.85 kb Sall-EcoRI-fragment was isolated from the plasmid pUC pyc by restriction with corresponding restriction enzymes as a 1.35 kb EcoRI-fragment, a 1.6 kb EcoRI-EcoRI-StuI-fragment as well as a 1.6 kb ClaI-fragment, that overlapped with 0.85 kb Sall-EcoRI-fragment. By ligation the fragments were cloned correspondingly in the restricting vector pUC 18 and then sequenced as described above according to Sanger et al.

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In (Proc Natl Acad Sci USA 1977, 74: 5463-5467) the nucleotide sequences obtained were analyzed. The program package HUSAR (Release 3.0) of the German zone for cancer research (Heidelberg). The sequence analysis of the fragments gave a continuously open reading raster of 3576 bp which coded for a protein sequence of 1140 amino acids. Comparison of the protein sequence with the EMBL gene data bank (Heidelberg) gave similarities to all known pyruvate carboxylases. The highest identity (62%) was to the putative pyruvate-carboxylase from *Mycobacterium tuberculosis* (EMBL- GeneBank: Accession No. U00024). The similarity amounted to 76% when conserved amino acid exchange was followed. A comparison with the pyruvate-carboxylase of other organisms yielded an identity of 46 to 47% identical and 64 to 65% similar amino acids (Gene 1997, 191: 47-50; J Bacteriol 1996, 178: 5960-5970; Proc Natl Acad Sci USA 1993, 90: 1766-1770; Biochem J 1996, 316: 631-637; EMBL- GenBank: Accession No. L36530; J Biol Chem 1988, 263: 11493-11497; Mol Gen Genet 1991, 229: 307-315). From these results it could be concluded that the cloned fraction base was the gene for the pyruvate-carboxylase from *C. glutamicum*. The nucleotide sequence of the gene is given under SEQ ID No. 1 and the corresponding amino acid sequence under SEQ ID No. 2.

3. Overexpression of the Pyruvate-Carboxylase

For the overexpression of the gene for pyruvate-carboxylase from *C. glutamicum*, the gene was cloned from the plasmid pUCpyc as the 6.2 kb SspI-ScaI-fragment in the *E. coli*

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glutamicum swing vector pEK0 (Gene 1991, 102: 93-98) which was cleaved with the restriction endonucleosis EcoRI and PstI. By means of Klenow-polymerase treatment the overhanging ends were ligated to smooth ends by filling the EcoRI or linking PstI and the linearized vector was ligated with the 6.2 kb SspI-ScaI-fragment. The resulting construct pEK0pyc was additionally transformed in the *E. coli* strain DH5 α , the plasmid DNA was isolated on the resulting transformand and the correctness of the inserts controlled by restriction. The DNA was then introduced in the strain SP 733 by electroporation (FEMS Microbiol Lett 1989, 65: 299-304).

This strain is a mutant of the restriction negative *C. glutamicum* strain R 127 (Dechema Biotechnology Conference 1990, 4: 323-327, Verlag Chemie) which was obtained by chemical mutagenesis and was characterized in that it cannot be grown on a minimal medium with pyruvate and lactate as single carbon sources (Microbiology 1997, 143: 1095-1103). This phenotype is recognized as a defect in the pyruvate-carboxylase and can be complemented by introducing the pyruvate-carboxylase gene from *C. glutamicum*, i.e. the strain which is carried by the plasmid pEK0pyc and was by contrast to the starting strain able to grow again in the presence of minimal medium with lactate as a single carbon source. This was a verification that the gene was coded for a functional pyruvate-carboxylase.

Furthermore, the plasmid pEK0pyc was transformed in the *C. glutamicum* wild type ATCC 13032 by electroporation. The resulting strain WT (pEK0pyc) was investigated by comparison to the

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wild type ATCC 13032 with respect to its pyruvate-carboxylase activity. The strain was cultured in a complex medium (Luria-Bertani, Molecular Cloning, A laboratory manual, 1989, Cold Spring Harbor Laboratory Press) with 0.5% lactate and on minimal medium with 2% lactate or 4% glucose and the pyruvate-carboxylase test was carried out corresponding to the method as described by Peters-Wendisch et al (Microbiology 1997, 143: 1095-1103). The results of the analysis (Table 1) showed that the pyruvate-carboxylase activity in the pEK0-*pyc*-carrying strain was about 4 times higher than in the starting strain.

4. Increased Accumulation of Lysine by Overexpression of the Pyruvate-Carboxylase Gene in the Strain *C-glutamicum* DG 52-5.

To investigate the effect of the overexpression of the gene for the pyruvate-carboxylase in the lysine-producing strain DG 52-5 (J Gen Microbiol 1988,134: 3221-3229), the expression vector pVWEX1 is used to promote an IPTG-inducible expression. In this vector, the *pyc* gene was promotorlessly cloned. For that purpose, initially PCT-Primer (Primer 1 = Position 112 - 133; Primer 2 = Position 373 to 355 in the nucleotide sequence according to SEQ ID No. 1), is synthesized and 261 bp of the promotorless starting region of the pyruvate-carboxylase gene was amplified by means of PCR. The primer was so selected that Primer 1 enabled a PstI cleavage site and Primer 2 a BamHI cleavage site. After the PCR, the 274 bp PCR product was isolated, ligated to concatemers and then cleaved with the restriction enzymes PstI and BamHI. The

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restriction product was concentrated by ethanol precipitation and then ligated with the PstI-BamHI cleaved vector pVWEX1. The resulting construct pVWEX1-PCR was tested by restriction. The end region of the pyc gene was isolated by RcaI-Klenow-SalI treatment from the vector pEK0pyc and ligated in the BamHI-Klenow-SalI during vector pVWEX1-PCR. The resulting construct pVWEX1pyc was analyzed by restriction mapping. Physical mapping of the plasmid is shown in FIG. 2.

The plasmid was introduced by electroporation in the *C. glutamicum* strain DG 52-5. As a control, the strain DG 52-5 was transformed with the vector pVWEX1 without insert and the L-lysine precipitation of three different transformants ^{WAS} ~~were~~ compared. For this purpose (DG 52-5 (pVWEX1pyc) 3,4 and (2xTY; Molecular Cloning, A laboratory manual, 1989, Cold Spring Harbour Laboratory Press with 50 µg/I kanamycin) and the respective fermentation medium in each case from the preculture was separately inoculated. The medium contained additional kanamycin to maintain the plasmid stable. In each case two parallel tests were run whereby one flask of 200 µg IPTG/ml was added while the second flask contained no IPTG. After cultivation for 48 hours at 30°C on a rotation shaker at 120 RPM, the accumulated lysine quantity in the medium was determined. The determination of the amino acid concentration was effected by means of high-pressure liquid chromatography (J Chromat 1983, 266; 471-482).

The results of the fermentation are shown in Table 2 whereby the values given are mean values each ^{from} ~~form~~ three

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experiments with different clones. It shows that the overexpression of the pyruvate-carboxylase gene results in a 50% increased accumulation of lysine in the medium. Thus the use of the covered and described gene for the anapleurotic enzyme pyruvate-carboxylase enables a process of lysine formation to be significantly improved.

5. Increased Accumulation of Threonine and Homoserine by Overexpression of the Pyruvate-Carboxylase Gene in the Strain *C. glutamicum* DM 368-3

Analogously to the experiment in L-lysine formation, the accumulation of threonine in the culture supernatant by overexpression of the gene for pyruvate-carboxylase was also investigated for this purpose, as has been described under point 4, the threonine production strain *C. glutamicum* DM 368-3 (Degussa AG) was transformed with the plasmid pVWEX1pyc with control by the plasmid pVWEX1 and the threonine separation was investigated with each of three different transformands. For this purpose DM 368-3 (pVWEX1) 2 and 3 and DM 368-3 (pVWEX1pyc) 1, 2 and 3 in complex medium (2xTY with 50 µg/l kanamycin) were cultured and the fermentation medium CGXII (J Bacteriol 1993, 175: 5595-5603) in each case was separately inoculated from the preculture. The medium contained additional kanamycin to hold the plasmid stable. Two parallel sets of tests were carried out whereby 200 µg IPTG/ml was added to one flask while the second flask contained no IPTG. After culturing for 48 hours at 30°C on a rotation shaker at 120

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RPM, the threonine quantities accumulated in the medium were determined. The determination of the amino acid concentration was effected also by means of high-pressure liquid chromatography (J Chromat 1983, 266: 471-482). The results of the fermentation are shown in Table 3 whereby the values given are mean values from each of three experiments with different clones. It shows that the overexpression of the pyruvate-carboxylase gene gave about a 40% increase in the threonine concentration in the medium. The use of the covered and described gene for anapleurotic enzyme pyruvate-carboxylase in a process for L-threonine formation significantly improves the latter.

Furthermore, the amino acid concentration determination shows surprisingly that the strain with the overexpressed pyruvate-carboxylase gene also yields 150% more homoserine in the medium than the strain with the nonoverexpressed gene. Corresponding results are shown in Table 3. They make clear that in the process according to the invention the threonine ^{yield} like the homoserine ^{yield} can be significantly improved. X

6. Increased Accumulation of Glutamate by Overexpression of the Pyruvate-Carboxylase Gene in *C. glutamicum* Wild Type

Analogous to the experiments for L-lysine, L-threonine and L-homoserine formation (see above, the 4. and 5.), accumulation of glutamate in the culture supernatant, overexpression of the gene for pyruvate-carboxylase was also investigated. For this purpose, as described, the point 4 wild type *C-glutamicum* ATCC 13032 with

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the plasmid pVWEX1 pyc was transformed in addition to the control with the plasmid pVWEX1 and the glutamate separation determined from each of two different transformands. Thus *C. glutamicum* ATCC 13032 pVWEX1pyc) D1 and D2 as well as *C. glutamicum* ATCC 13032 (pVWEX1 pyc) 1 and 2 were cultured in the complex medium (2xTY with 50 µg/l kanamycin) and the fermentation medium CGXII (J Bacteriol 1993, 175: 5595-5603) in each case was separately inoculated from the preculture period. The medium contained additional kanamycin to stabilize the plasmid. To induce glutamate separation, 25 mg Tween 60 was added per ml to the medium about 6 hours after the inoculation. Two parallel sets of tests were carried out whereby in one, 200 µg IPTG/ml is added to the flask while the second flask contained no IPTG. After culturing for 48 hours at 30°C on a rotation shaker at 120 RPM, the glutamate quantity accumulated in the medium was determined. The determination of the amino acid concentration was effected also by means of high-pressure liquid chromatography (J Chromat 1983, 266; 471-482). The results of the fermentation are shown in Table 4 whereby values given are averages with each two experiments with different clones. It shows that the overexpression of the pyruvate-carboxylase gene gave rise to up to 500% increase of the glutamate concentration in the medium. The use of the covered and described gene for the anapleurotic enzyme pyruvate-carboxylase improved the glutamate formation significantly.

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Strain	IPTG [$\mu\text{g/ml}$]	Pyruvate Carboxylase [nmol min ⁻¹ mg Dry Weight ⁻¹]
13032(pEK0pyc)	0	75 \pm 13
ATCC 13032	0	19 \pm 4
DG52-5(pVWEX1pyc)	200	88 \pm 13
	0	11 \pm 2
DG52-5(pVWEX1)	200	5 \pm 2
	0	6 \pm 1
DM368-3(pVWEX1pyc)	200	76 \pm 10
	0	12 \pm 3
DM368-3(pVWEX1)	200	10 \pm 1
	0	11 \pm 2

Table 1

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Strain	IPTG [μ g/ml]	Lysine [mM]
DG52-5(pVWEX1pyc)	200	35.4 \pm 2.6
	0	23.6 \pm 2.9
DG52-5(pVWEX1)	200	23.3 \pm 2.9
	0	22.1 \pm 4.0

Table 2

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Strain	IPTG [µg/ml]	Threonine [mM]	Homoserine [mM]
DM368-3(pVWEX1pyc)	200	10.2 ± 0.5	14.4 ± 1.2
	0	7.9 ± 1.0	5.6 ± 0.2
DM368-3(pVWEX1)	200	8.0 ± 0.5	5.8 ± 0.7
	0	7.5 ± 0.8	6.1 ± 1.0

Table 3

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Strain	IPTG [μ g/ml]	Glutamate [mM]
ATCC 13032	200	11 \pm 2
ATCC 13032	0	13 \pm 2
ATCC 13032(pVWEX1-pyc)	200	67 \pm 4
ATCC 13032(pVWEX1-pyc)	0	32 \pm 4

Table 4

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~~Patent Claims~~

WHAT IS CLAIMED IS:

1 1. A method of microbial production of amino acids of
2 aspartate and/or glutamate families in which the pyruvate-
3 carboxylase activity is increased by genetic modification of the
4 enzyme and/or the pyruvate-carboxylase gene expression of the
5 corresponding amino-acid-producing micro organism.

1 2. The method of claim 1, characterized in that, by
2 mutation of the endogenous pyruvate-carboxylase gene an enzyme with
3 higher pyruvate-carboxylase activity is produced.

1 3. The method of claim 1 (or 2), characterized in that,
2 the gene expression of the pyruvate-carboxylase is increased by
3 increasing the gene copy number.

1 4. The method according to claim 3, characterized in
2 that, to increase the gene copy number the pyruvate-carboxylase
3 gene is incorporated in a gene construct.

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1 ~~5~~. The method according to claim 3, characterized in
2 that, the gene is incorporated in a gene construct which contains
3 regulatory gene sequences associated with the pyruvate-carboxylase
4 gene.

1 ~~6~~. The method according to claim 4 (or 5), characterized
2 in that, the corresponding amino-acid-producing microorganism is
3 transformed with the gene-containing gene construct.

1 ~~7~~. The method according to claim 6, characterized in
2 that, a microorganism of the species *Corynebacterium* is transformed
3 with the gene containing the gene construct.

1 ~~8~~. The method according to claim 6 (or 7), characterized
2 in that, for the transformation a microorganism is used in which
3 the enzyme participating in the synthesis of the corresponding
4 amino acid is deregulated and/or wherein an enhanced export carrier
5 activity is shown for the corresponding amino acid.

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1 ~~9.~~ The method according to claim 6 (to 8), characterized
2 in that, for the transformation a microorganism is used which has a
3 higher proportion of the central metabolism metabolites of the
4 corresponding amino acid participating in the synthesis.

1 ~~10.~~ The method according to claim 6 (to 9), characterized
2 in that, for the transformation a microorganism is used in which
3 biosynthesis paths competing with the corresponding amino acid
4 biosynthesis paths runs with reduced activity.

1 ~~11.~~ The method according to (one of the preceding claims),
2 characterized in that, the pyruvate-carboxylase gene is isolated
3 from a microorganism strain of the variety *Corynebacterium*.

1 ~~12.~~ The method according to (one of the preceding claims),
2 characterized in that, the gene expression is increased by
3 reinforcement of the transcription signal.

1 ~~13.~~ The method according to (one of the preceding claims),
2 characterized in that, the pyruvate-carboxylase gene has the tac-
3 promoter ahead of the pyruvate-carboxylase gene.

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1 ~~14.~~ The method according to claim 13, characterized in
2 that, the tac-promoter is associated with regulatory sequences.

1 ~~15.~~ The method according to *cl 1* (one of the preceding claims),
2 characterized in that, the pyruvate-carboxylase gene is a gene with
3 the amino acid sequence given under SEQ ID No. 2 and its allele
4 variation coding nucleotide sequences.

1 ~~16.~~ The method according to claim 15, characterized in
2 that, with the pyruvate-carboxylase gene a gene with the nucleotide
3 sequence of nucleotide 165 to 3587 according to SEQ ID No. 1 or a
4 substantially identically-effective DNA sequence is used.

1 ~~17.~~ The method according to *cl 1* (one of the preceding claims)
2 for the production of lysine, threonine, homoserine, glutamate
3 and/or arginine.

1 ~~18.~~ A pyruvate-carboxylase gene coding for the amino
2 acid sequence given under SEQ ID No. 2 and /or a nucleotide
3 sequence coding for its allele variations.

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1 ~~19.~~ The pyruvate-carboxylase gene according to claim 18
2 with the nucleotide sequence of nucleotides 165 to 3587 according
3 to SEQ ID No. 1 or a substantially identically-effective DNA
4 sequence.

1 ~~20.~~ The pyruvate-carboxylase gene according to claim 18
2 (or 19) with a preceding promoter of the nucleotide sequence from
3 nucleotide 20 to 109 according to SEQ ID No. 1 or a substantially-
4 identically-effective DNA sequence.

5 ~~21.~~ The pyruvate-carboxylate gene according to claim 18
6 (or 19), with preceding tac-promoter.

7 ~~22.~~ The pyruvate-carboxylase gene according to claim 21
8 with the regulatory sequence associated with the promoter.

1 ~~23.~~ The pyruvate-carboxylase gene according to (one of
2 claims 18 to 20) with these regulatory gene sequences associated
3 therewith.

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1 ~~24.~~ A gene structure containing a pyruvate-carboxylase
2 gene according to ^{cl 18}(one of claims 18 to 23).

3 ~~25.~~ A vector containing a pyruvate-carboxylase gene
4 (according to one of claims 18 to 23) or a gene structure according
5 to claim ¹⁸(24).

1 ~~26.~~ Transformed cells containing in replicatable form a
2 pyruvate-carboxylase gene (according to one of claims 18 to 23) or a
3 gene structure according to claim (24).

1 ~~27.~~ Transformed cells (according to claim 26) containing a
2 vector according to claim 25.

1 ~~28.~~ Transformed cells according to claim 26 (or 27),
2 characterized in that, they belong to the variety *Corynebacterium*.

1 ~~29.~~ Transformed cells according to ^{cl 26}(one of claims 26 to
2 28), characterized in that, enzymes which participate in the
3 synthesis of the corresponding amino acid and/or enzyme which
4 participate in the export of the corresponding amino acid are
5 deregulated.

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cl 26

6 ~~30.~~ Transformed cells according to (one of claims 26 to
7 29) characterized in that, they contain an increased proportion of
8 the central metabolism metabolites participating in the synthesis
9 of the corresponding amino acid.

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1 ~~31.~~ Transformed cells according to (one of claims 26 to
2 30), characterized in that, they contain a reduced proportion of the
3 central metabolism metabolites which do not participate in the
4 synthesis of the corresponding amino acid.

1 ~~32.~~ The use of a pyruvate-carboxylase gene for
2 increasing the production of amino acids of the aspartate and/or
3 glutamate families by microorganisms.

1 ~~33.~~ The use according to claim 32, characterized in
2 that, a mutated pyruvate-carboxylase gene which codes for an enzyme
3 with increase pyruvate-carboxylase activity is used.

1 ~~34.~~ The use according to claim 32 (or 33), characterized
2 in that, the microorganism producing the corresponding amino acid

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3 is transformed with a gene construct that contains a pyruvate-
4 carboxylase gene.

1 ~~35.~~ The use according to claim 34, characterized in
2 that, the gene construct additionally contains regulatory gene
3 sequences.

1 ~~36.~~ The use according to (one of claims 32 or 35),
2 characterized in that, a pyruvate-carboxylase gene from
3 *Corynebacterium* is used.

1 ~~37.~~ The use according to (one of claims 32 or 36),
2 characterized in that, *Corynebacterium* is used as the amino acid-
3 producing microorganism.